

## DESCRIPTION

ANTIBODY AGAINST NOX1 POLYPEPTIDE, DIAGNOSTIC METHOD FOR  
CANCER USING NOX1 GENE, AND SCREENING METHOD FOR CANCER  
5 GROWTH INHIBITOR

## Technical Field

The present invention relates to a diagnostic method for cancer, a screening method  
for a cancer growth inhibitor, and a pharmaceutical composition based on a  
10 cancer-associated gene and a polypeptide coded for the gene. More specifically, the  
present invention relates to a diagnostic method for cancer; a screening method for a  
cancer growth inhibitor; and a pharmaceutical composition used in cancer therapy, using a  
Nox1 gene associated with a mutant Ras oncogene, a polypeptide coded for the gene, and  
an antibody specific to the polypeptide.

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## Background Art

A Mutant Ras oncogene is heretofore known to exert great influence on mammalian cell  
carcinogenesis and its progression (Non-Patent Document 1). For example, the mutant  
Ras oncogene is considered to cancerate animal cells and aggravate the cancer via a  
20 Raf-MAPKK-MAPK pathway, one of its principal downstream pathways (Non-Patent  
Document 2).

Based on these findings, there have been developed a method for tumor suppressor  
gene therapy comprising administering an expression vector gene comprising a gene  
coding for p94RB to a targeted cancer cell depleted of N-ras tumor suppressor (Patent  
25 Document 1), and a method of identifying a tumor suppressor gene by use of H-ras, K-ras,  
and N-ras oncogenes (Patent Document 2).

Recent research has reported that cells transformed with mutant Ras gene produce  
reactive oxygen species (ROS) such as superoxide and H<sub>2</sub>O<sub>2</sub> (Non-Patent Document 3).  
Low-level intracellular ROS plays a role as a signal molecule in growth factor-induced cell  
30 growth (Non-Patent Documents 4 and 5), suggesting that a rise in ROS generation  
associated with mutant Ras gene possibly causes the abnormal growth of animal cells.

However, reactive oxygen-producing enzymes that are involved in carcinogenesis,  
cancer promotion, and so on, caused by mutant Ras gene have heretofore been unknown.

(Patent Document 1) National Publication of International Patent Application No. 1996-508166

(Patent Document 2) National Publication of International Patent Application No. 1998-504448

5 (Non-Patent Document 1) Lowy, D. R. Annu. Rev. Biochem. 62, 851-891 (1993)

(Non-Patent Document 2) McCormick, F. TCB. 9, M53-M56 (1999)

(Non-Patent Document 3) Irani, K. et al. Science 275, 1649-1652 (1997)

(Non-Patent Document 4) Sundaresan, M. et al. Science. 270, 296-299 (1995)

(Non-Patent Document 5) Bae, Y. S. et al. J. Biol. Chem. 272, 217-221 (1997)

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#### Disclosure of the Invention

An object of the present invention is to provide a diagnostic method for cancer and a screening method for a cancer growth inhibitor; and a pharmaceutical composition used in cancer therapy using a Nox-1 gene associated with a mutant Ras oncogene, a  
15 polypeptide coded by the gene, and an antibody specific to the polypeptide.

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The present inventors conducted studies and consequently gained findings that a mutant Ras oncogene remarkably increases the expression of Nox1 (1, 2, and 3), a homologue of the catalytic subunit of superoxide-generating NADPH oxidase, in an MAPKK-MAPK pathway, and that siRNA of the Nox1 gene effectively suppresses  
20 phenotypes of a mutant Ras gene such as cell adhesion-dependent growth, morphological changes, and tumor formulation in athymic mice. Based on the findings, the present inventors attained the object and completed the present invention.

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Namely, the present invention provides a composition for producing an antibody, comprising: (1) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2; (2) a  
25 polypeptide having an amino acid sequence mutated from the amino acid sequence of SEQ ID NO: 2 by the substitution, deletion, addition, and/or insertion of one or more amino acid residues and inducing the production of an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2; or (3) a polypeptide fragment having a partial sequence of the polypeptide of (1) or (2) and inducing the production of an antibody  
30 specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2.

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Moreover, the present invention provides a method for producing an antibody specific to a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, comprising administering the composition for producing an antibody to a mammal.

Furthermore, the present invention provides an antibody specific to a polypeptide comprising the amino acid sequence of SEQ ID NO: 2.

Furthermore, the present invention provides a diagnostic method for cancer, comprising bringing the antibody specific to a polypeptide comprising the amino acid  
5 sequence of SEQ ID NO: 2 into contact with a biological sample.

Furthermore, the present invention provides a diagnostic composition for cancer and a pharmaceutical composition for cancer therapy, comprising the antibody.

Furthermore, the present invention provides a diagnostic method for cancer, characterized by detecting a polynucleotide comprising the nucleotide sequence of SEQ ID  
10 NO: 1 or a fragment thereof. It is preferred that the detection should be performed by detecting the polynucleotide or the fragment thereof by polymerase chain reaction or real-time quantitative polymerase chain reaction.

Furthermore, the present invention provides siRNA corresponding to a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment  
15 thereof.

Furthermore, the present invention provides a pharmaceutical composition for cancer therapy, comprising: siRNA corresponding to a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof; and siRNA corresponding to a polynucleotide comprising a nucleotide sequence at positions from 71 to 1615 of SEQ ID  
20 NO: 1 or a fragment thereof.

Furthermore, the present invention provides a pharmaceutical composition for cancer therapy, comprising a cell transformed with siRNA corresponding to a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof; and siRNA corresponding to a polynucleotide comprising a nucleotide sequence at  
25 positions from 71 to 1615 of SEQ ID NO: 1 or a fragment thereof.

Furthermore, the present invention provides a screening method for a cancer cell growth inhibitor targeted to a Nox1 gene, comprising: (1) transfecting a cell with a Nox1 gene; bringing the transformed cell into contact with a substance to be screened; and detecting the inactivation of Nox1 activity (primary screening); and (2) investigating  
30 whether or not the Nox1-inactivating substance suppresses cancer cell growth (secondary screening), thereby detecting the expression of the Nox1 gene and the inactivation of Nox1 activity.

(Definition)

The term “polypeptide” used herein means a primary structure of an amino acid sequence, a polypeptide fragment with the primary structure, a polypeptide having biological activity accompanied with a three-dimensional structure, or a protein.

5 A “Nox1 polypeptide” used herein refers to a polypeptide coded for a Nox1 gene and includes both full-length polypeptide and polypeptide fragment.

The term “homologue” used herein refers to a polypeptide having homology in an amino acid sequence to a polypeptide or protein with a particular amino acid sequence and having biological activity or antigenicity in common with the polypeptide or protein.

10 The term “siRNA” used herein means a short RNA fragment that suppresses the expression of a particular gene or a double-stranded RNA molecule that consists of the RNA fragment and its complementary strand.

A “mutant Ras gene” used herein refers to a human Ras gene in a mutated state that is involved in tumor formation. The mutant Ras gene is, for example, a human Ras gene having a K-Ras mutation of glycine at the 12th codon to aspartic acid or to valine or  
15 arginines, which is commonly seen in human pancreatic cancer, as well as a human Ras gene having K-Ras mutations occurring at the 12th codon and additionally at the 13th and 61st codons, which are found in lung cancer.

A “partial sequence of an amino acid sequence” of the polypeptide used herein refers to a sequence that comprises at least 8 or more consecutive amino acid residues  
20 contained in the amino acid sequence.

An “oligonucleotide” used herein refers to a nucleotide that typically comprises less than 100 bases and preferably comprises 6 to 99 bases.

#### Brief Description of the Drawings

25 Fig. 1(a) is a graph showing a result of gene expression analysis conducted by real-time quantitative polymerase chain reaction in Example 1;

Fig. 1(b), (c), and (d) are, results of detecting Nox1 expression in Example 2 by conducting real-time quantitative polymerase chain reaction in the same way as in Example 1;

30 Fig. 2 is an electrophoretic image obtained in Example 4 by conducting PCR by use of M13F and 3.0Rev as primers to confirm a result of transformation of cell lines stably transfected with RNAi(1), RNAi(3), or RNAi(5);

Fig. 3 shows photographs obtained in Example 4, wherein morphological changes in which Nox1 gene is involved are compared among cells;

Fig. 4 is a graph showing a result of measuring the adhesion-dependent cell growth of cell lines exhibited by soft agar culture in Example 4 in order to examine the transformation of cells by observing anchorage-independent growth capacity;

Fig. 5 is a graph showing a growth curve obtained in Example 4 by measuring the number of each of K-Ras-NRK/neg-1 (neg-1), K-Ras-NRK/RNAi(1)-7 (i(1)-7), K-Ras-NRK/RNAi(3)-19 (i(3)-19), and K-Ras-NRK/RNAi(5)-7 (i(5)-7) cells in a liquid medium;

Fig. 6 is a graph showing a result of quantifying a decrease in NBT in Example 5 by solubilizing treated cells and measuring absorbance at 510 nm;

Fig. 7 is a graph showing in Example 6 that a GFP-rat Nox1 fusion polypeptide is produced, that the coexpression of RNAi(1), RNAi (3), and RNAi(5) inhibits the production of a GFP-rat Nox1 fusion protein dose-dependently on their vectors (Left panel), and that the RNAi constructs have high specificity to their target gene (right panel);

Fig. 8 is an electrophoretic image obtained in Example 6 by conducting RT-PCR to confirm that the mRNA expression of endogenous Nox1 is inhibited in each of cells, as compared to K-Ras-NRK and K-Ras-NRK/neg-1 cells;

Fig. 9 is an image obtained in Example 7 by immunoblotting by the same approach as in Example 6, wherein the image demonstrates that siRNA used does not inhibit GFP or GFP-human Nox1 expression;

Fig. 10 is a photograph showing the influence of reactivation of Nox1 once inhibited by siRNA on cells in terms of morphological changes in K-Ras-NRK cells in Example 7;

Fig. 11 is a graph showing the influence of reactivation of Nox1 once inhibited by siRNA on cells in terms of cell growth rates;

Fig. 12 is a graph showing a result of investigating adhesion-dependent cell growth capacity by use of a soft agar culture method in order to examine the influence of reactivation of Nox1 once inhibited by siRNA on cells by observing anchorage-independent growth capacity;

Fig. 13 is a graph showing a result of observing morphological changes in cells in order to investigate the influence of Nox1 gene expression on transformation with mutant Ras in Example 9; and

Fig. 14 is a graph showing the influence of siRNA on tumor formation caused by mutant Ras by observing tumor formation in a nude mouse over 1 month and measuring the volume of the formed tumor in Example 9.

5 Most Preferable Mode for Carrying Out the Invention

A composition for producing an antibody of the present invention comprises: (1) a polypeptide comprising the amino acid sequence of SEQ ID NO: 2; (2) a polypeptide having an amino acid sequence mutated from the amino acid sequence of SEQ ID NO: 2 by the substitution, deletion, addition, and/or insertion of one or more amino acid residues  
10 and inducing the production of an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2; or (3) a polypeptide fragment having a partial sequence of the polypeptide of (1) or (2) and inducing the production of an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2.

The composition for producing an antibody of the present invention may contain a  
15 polypeptide having at least 80% homology, preferably 90% homology, particularly preferably 95% homology, to the amino acid sequence of SEQ ID NO: 2. The homology of amino acid sequences used herein refers to identity (%) between a reference amino acid sequence and an amino acid sequence compared therewith when the sequences are aligned. The homology can be calculated using default (initial setting) parameters in analytical  
20 software such as BLAST (J. Mol. Biol., 215, 403 (1990)) and FASTA (Methods in Enzymology, 183, 63-69) that are standard programs for performing the homology search of nucleotide sequences and amino acid sequences.

The polypeptide comprising the amino acid sequence of SEQ ID NO: 2 is a polypeptide coded for a Nox1 gene. The carcinogenesis and cancer progression of tissue  
25 to be examined can be screened by detecting the production of the polypeptide. Although an antibody manufactured with the composition is specific to a human Nox1 polypeptide, the antibody can also be used in the screening of the carcinogenesis and cancer progression of non-human animals when having specificity to homologues of the polypeptide produced by the non-human animals. Thus, the composition for producing an antibody can also be  
30 used for producing an antibody for detecting a human cancer cell and an antibody for screening the carcinogenesis and cancer progression of mammals capable of producing human Nox1 polypeptide homologues.

The partial sequence of the amino acid sequence of SEQ ID NO: 2 is a sequence that comprises at least 8 or more consecutive amino acid residues contained in the amino acid sequence and refers to any of those serving as an epitope that induces the production of an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2. When the partial sequence is used as an epitope, it can be used in combination with an appropriate carrier protein.

The polypeptide fragment of the present invention is any fragment of the polypeptides (1) and (2) and induces the production of an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2.

The length of the peptide fragment is not particularly limited and however, is typically 20 to 200 amino acid residues, preferably 20 to 100 amino acid residues, more preferably 20 to 70 amino acid residues, in length.

In the present specification, the N-terminuses (amino terminuses) of the polypeptide, the homologue, and the polypeptide fragment (hereinafter, abbreviated to the polypeptide, if necessary) are indicated in the left, and the C terminuses (carboxyl terminuses) thereof are indicated in the right, in accordance with the standard notation. The polypeptide and so on, of the present invention can have a carboxyl group (-COOH), carboxylate (-COO-), amide (-CONH<sub>2</sub>), or ester (-COOR) at the C terminus. Examples of the side chain R of this ester include: C<sub>1</sub> to C<sub>6</sub> alkyl groups such as methyl, ethyl, n-propyl, isopropyl, and n-butyl; C<sub>3</sub> to C<sub>8</sub> cycloalkyl groups such as cyclopentyl and cyclohexyl; C<sub>6</sub> to C<sub>12</sub> aryl groups such as phenyl and  $\alpha$ -naphthyl; and alkyl groups such as phenyl-C<sub>1</sub> to -C<sub>2</sub> alkyl groups such as benzyl and phenethyl or  $\alpha$ -naphthyl-C<sub>1</sub> to -C<sub>2</sub> alkyl groups such as  $\alpha$ -naphthylmethyl.

The polypeptide and so on, of the present invention also includes: those in which an amino group of an N-terminal amino acid residue is protected with a protecting group such as a formyl group and an acetyl group; those in which an N-terminal glutamine residue generated in vivo by cleavage is converted to pyroglutamic acid; those in which a substituent (e.g., -OH, -SH, an amino group, an imidazole group, an indole group, or a guanidino group) on the side chain of an intramolecular amino acid is protected with a suitable protecting group; or a conjugated protein such as so-called glycoprotein bound with a sugar chain.

Moreover, the polypeptide and so on, of the present invention can be used as a physiologically acceptable inorganic or organic acid-addition salt. Examples of the

inorganic acid-addition salt include salts of hydrochloric acid, phosphoric acid, hydrobromic acid, and sulfuric acid, and examples of the organic acid-addition salt include salts of acetic acid, formic acid, propionic acid, fumaric acid, maleic acid, succinic acid, tartaric acid, citric acid, malic acid, oxalic acid, benzoic acid, methanesulfonic acid, and  
5 benzenesulfonic acid.

Namely, the composition for producing an antibody of the present invention comprises the polypeptide, the homologue thereof, and their peptide fragments, or their acid-addition salts. The composition may optionally contain ingredients such as a vehicle, a diluent, and an adjuvant.

10 The present invention provides a method of producing an antibody specific to the polypeptide comprising the amino acid sequence of SEQ ID NO: 2, the homologue thereof, or the peptide fragment by use of the composition for producing an antibody.

An antibody manufactured by the method of the present invention is not particularly limited as long as it can be used according to the object of the present invention.

15 Examples of the antibody include a human/mouse chimeric antibody, a humanized antibody, or a human antibody. In this context, the humanized antibody refers to any of those containing several percent of a mouse-derived antibody in the whole antibody, while the human antibody refers to an antibody derived 100% from a human. Meanwhile, the chimeric human antibody refers to any of those containing 10 to 20% of a mouse-derived  
20 antibody. The antibody may be any of polyclonal and monoclonal antibodies.

The monoclonal antibody of the present invention can be manufactured by the following procedures: at first, the composition for producing an antibody is administered to non-human mammals. A complete Freund's adjuvant or incomplete Freund's adjuvant may be administered for enhancing the ability of the mammals to produce antibodies.

25 The administration is typically performed once every 2 to 6 weeks with a total of approximately 2 to 10 doses. Examples of the non-human mammals include monkeys, rabbits, dogs, guinea pigs, mice, rats, sheep, goats, and chickens, with mice and rats generally preferred.

An individual in which antibody production has been observed is selected from the  
30 non-human mammals immunized with the composition. Two to Five days after final immunization, antibody-producing cells contained in the spleen or lymph node are collected from the individual and fused with myeloma cells from animals of the same or different species to thereby create monoclonal antibody-producing hybridomas. Antibody



titer in antiserum is measured by allowing a labeled protein to react with the antiserum and then measuring the activity of the labeling agent bound with the antibody. Cell fusion can be performed by a routine method such as the method of Kohler and Milstein (Nature, 256, 495, 1975). For example, polyethylene glycol (PEG) or a Sendai virus can be used as a fusion promoter.

Examples of the myeloma cells include non-human mammalian myeloma cells such as NS-1, P3U1, SP2/O, and AP-1, with P3U1 particularly preferred. A preferable ratio of the number of the antibody-producing cells (spleen cells) to the number of the myeloma cells, used in the fusion, is approximately 1:1 to 20. The spleen and myeloma cells can be fused by incubation at 20 to 40°C, preferably 30 to 37°C, for 1 to 10 minutes, with PEG (preferably PEG1000 to PEG6000) added at a concentration of approximately 10 to 80%.

The monoclonal antibody-producing hybridomas can be screened by a variety of methods. Examples of the methods include: a method that involves bringing a hybridoma culture supernatant into contact with a solid phase (e.g., a microplate) where an antigen is adsorbed either directly or in combination with a carrier and subsequently bringing a solution containing an anti-immunoglobulin antibody labeled with a radioactive substance, an enzyme, or the like, into contact therewith to detect the monoclonal antibody bound to the solid phase; and a method that involves bringing a hybridoma culture supernatant into contact with a solid phase where an anti-immunoglobulin antibody or the like is adsorbed and subsequently bringing a solution of a protein labeled with a radioactive substance, an enzyme, or the like, into contact therewith to detect the monoclonal antibody bound to the solid phase.

The monoclonal antibody-producing hybridomas can be selected by a routine method. For example, a medium for animal cells supplemented with HAT (hypoxanthine-aminopterin- thymidine), an RPMI 1640 medium containing 10 to 20% fetal bovine serum, a GIT medium containing 1 to 10% fetal bovine serum (Wako Pure Chemical Industries), or a serum-free medium for culturing hybridomas (SFM-101; Nissui Pharmaceutical) can be used. A culture temperature is 20 to 40°C, and a culture time is typically 5 days to 3 weeks. The culture can typically be performed under 5% carbonic acid gas. Antibody titer in the hybridoma culture supernatant can be measured in the same way as in the above-described measurement of antibody titer in antiserum.

The obtained monoclonal antibody can be separated and purified by a routine method such as: an immunoglobulin separation and purification method such as salting-out,

alcohol precipitation, isoelectric precipitation, and electrophoresis; an absorption and desorption method with an ion exchanger (DEAE); an ultracentrifugation method; a gel filtration method; an antigen-bound solid phase; or a specific purification method performed by collecting only an antibody with a protein A active adsorbent and then  
5 dissociating the bond to obtain the antibody.

Alternatively, the polyclonal antibody of the present invention can be manufactured by immunizing non-human mammals with the polypeptide and so on serving as an immunizing antigen or a complex of the peptide fragment thereof with a carrier protein and then collecting an antibody-containing component such as serum therefrom, followed by  
10 antibody separation and purification.

The mixing ratio of the immunizing antigen to the carrier protein is determined so that the antibody can efficiently be raised against the hapten that has been crosslinked with the carrier and used in immunization. For example, approximately 0.1 to 20 parts by weight, preferably approximately 1 to 5 parts by weight, of bovine serum albumin, bovine  
15 thyroglobulin, or hemocyanin can be used with respect to 1 part by weight of the hapten. For example, glutaraldehyde, carbodiimide, maleimide-activated ester, and activated ester reagents containing a thiol or dithiopyridyl group can be employed in the coupling between the hapten and the carrier.

The complex antigen may be administered alone or in combination with a carrier or  
20 diluent, or alternatively, for enhancing the ability of the mammals to produce antibodies, with a complete Freund's adjuvant or incomplete Freund's adjuvant. The administration is typically performed once every 2 to 6 weeks with a total of approximately 3 to 10 doses. The polyclonal antibody is collected from the blood, ascites, yolk, or the like, of the immunized mammal. Polyclonal antibody titer in antiserum can be measured in the same  
25 way as in the above-described measurement of antibody titer in antiserum. The polyclonal antibody can be separated and purified in the same way as in the above-described separation and purification of the monoclonal antibody.

A diagnostic kit for cancer of the present invention comprises the polyclonal or monoclonal antibody thus obtained. The diagnostic kit optionally contains wells for  
30 immune reaction, a staining agent, an enzyme-labeled antibody for detection, a cleansing liquid, an antibody diluent, a sample diluent, an enzyme substrate, a diluent of an enzyme substrate solution, and additional reagents.

A pharmaceutical composition comprising the antibody of the present invention suppresses the expression of a specific protein coded for a Nox1 gene and as such, can be employed in the prevention, delayed progression, and treatment of carcinogenesis.

The antibody can be administered orally or parenterally, and the parenteral  
5 administration includes local administration to tissue.

When the pharmaceutical composition of the present invention is orally administered, pharmaceutical ingredients such as carriers widely used, for example, fillers, expanders, binders, disintegrants, disintegration inhibitors, buffers, tonicity agents, emulsifiers, dispersants, stabilizers, coating agents, surfactants, absorption promoters,  
10 humectants, wetting agents, adsorbents, lubricants, and excipients can be used. Additives such as coloring agents, preservatives, perfumes, flavoring agents, and sweetening agents may optionally be used.

Concrete examples of the pharmaceutical ingredients include: excipients such as milk sugar, white sugar, sodium chloride, grape sugar, urea, starch, calcium carbonate,  
15 kaoline, crystalline cellulose, and silicic acid; binders such as water, ethanol, simple syrup, grape sugar fluids, starch fluids, gelatin solutions, carboxymethylcellulose, shellac, methylcellulose, potassium phosphate, and polyvinylpyrrolidone; disintegrants such as dried starch, sodium alginate, agar powder, laminaran powder, sodium hydrogencarbonate, calcium carbonate, polyoxyethylene sorbitan fatty acid ester, sodium lauryl sulfate, stearic  
20 acid monoglyceride, starch, and milk sugar; disintegration inhibitors such as white sugar, stearic acid, cacao butter, and hydrogenated oil; absorption promoters such as quaternary ammonium salts and sodium lauryl sulfate; humectants such as glycerin and starch; adsorbents such as starch, milk sugar, kaolin, bentonite, and colloidal silicic acid; and lubricants such as purified talc and stearate. In addition, the technique of a drug delivery  
25 system known in the art is optionally adopted for each of the dosage forms to provide for the sustained release, local application (e.g., troches, buccals, and sublingual tablets), drug release control, conversion to an enteric coated form, conversion to a gastrolytic form, and so on, of the pharmaceutical composition.

When the pharmaceutical composition of the present invention is parenterally  
30 administered, the pharmaceutical composition can be used in forms such as administration by injection such as instillation, intravenous injection, hypodermic injection, and intramuscular injection, and endorectal administration with suppositories, for example, suppositories made of fats and oils and water-soluble suppositories. The pharmaceutical

composition in such a form can be prepared with ease by a routine method using a typical carrier in the pharmaceutical field.

The pharmaceutical composition comprising the antibody of the present invention can be administered to, for example, humans or other mammals (e.g., rats, mice, guinea  
5 pigs, rabbits, sheep, pigs, cattle, horses, cats, dogs, and monkeys). The amount of the pharmaceutical composition administered appropriately differs depending on the conditions of objects to be administered, administration routes, and so on. For example, when orally administered, the pharmaceutical composition is generally administered at approximately 10 to 4000 mg, preferably approximately 20 to 2000 mg, more preferably  
10 approximately 50 to 500 mg, per day for an adult patient weighing 60 kg. When parenterally administered, it is preferred that the siRNA, for example in the form of injection, should be administered through the vein at a dose of approximately 10 to 2000 mg, preferably approximately 20 to 1000 mg, more preferably approximately 50 to 500 mg, per day for an adult patient weighing 60 kg although the dose differs depending on objects  
15 to be administered, the condition of liver cancer, and so on.

Cancer diagnosis can be performed by detecting and quantifying a Nox1 gene in biological tissue or bodily fluids by immunochemical assay comprising bringing the antibody of the present invention into contact with a biological sample.

When this immunochemical assay is conducted, the antibody of the present  
20 invention is held in a carrier. Examples of the carrier used in the assay include: gel particles such as agarose gels (e.g., Sepharose 4B and Sepharose 6B; Pharmacia Fine Chemicals), dextran gels (e.g., Sephadex G-75, Sephadex G-100, and Sephadex G-200; Pharmacia Fine Chemicals), polyacrylamide gels (Bio-Gel P-30, Bio-Gel P-60, and Bio-Gel P-100; BioRad Laboratories), and cellulose particles (Avicel; Asahi Kasei);  
25 ion-exchange cellulose such as diethylaminoethylcellulose and carboxymethylcellulose; physical adsorbents such as glass, silicone strips, and stainless steel-based resins; immunoassay plates (Nunc); and weakly basic anion-exchange resins (e.g., Amberlite IR-4B and DOWEX-3; Dow Chemical).

The antibody can be held in the carrier by a routine method such as cyanogen  
30 bromide and glutaraldehyde methods. In a more convenient way, the carrier may be adsorbed physically onto the surface of the antibody. Examples of a labeling agent to be bound to the antibody include radioactive isotopes, enzymes, fluorescent substances, and luminescent substances, with enzymes preferably used.

Any of those being stable and having high specific activity is preferable as the enzyme, and peroxidase, alkaline phosphatase,  $\beta$ -D-galactosidase, and glucose oxidase can be used, with peroxidase particularly preferred.

5 A sample to be tested in the immunochemical assay system of the present invention is a bodily fluid such as urine, serum, plasma, and spinal fluid, or an animal cell or a culture supernatant thereof. When the immunochemical assay of the present invention is conducted, a substance to be analyzed such as a Nox1 polypeptide to be measured is added to the antibody held in the carrier to perform antigen-antibody reaction. Then, the bound substance of an anti-Nox1 antibody and the labeling agent is further added thereto and  
10 reacted. The resulting reaction product is supplemented with the labeling agent (e.g., substrate of enzyme). The enzyme activity of the reaction product can be determined by measuring the absorbance or fluorescence intensity of a generated substance. These procedures are performed in advance on a standard solution of a known amount of the labeling agent (e.g., enzyme) to construct a standard curve that plots the relationship of  
15 absorbance or fluorescence intensity versus the amount of the Nox1 polypeptide or the like. This standard curve is compared with the absorbance or fluorescence intensity obtained from the substance to be analyzed (sample to be tested) comprising an unknown amount of the Nox1 polypeptide to measure the amount of the Nox1 polypeptide in the substance to be analyzed.

20 Moreover, the present invention provides a diagnostic method for cancer, characterized by detecting a polynucleotide comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof. The polynucleotide or the fragment thereof can be detected by a routine method utilizing polymerase chain reaction (PCR) or real-time quantitative polymerase chain reaction.

25 The polymerase chain reaction or the real-time quantitative polymerase chain reaction uses a sense strand fragment corresponding to the nucleotide sequence of SEQ ID NO: 1 as a forward primer and an antisense strand fragment corresponding to the nucleotide sequence of SEQ ID NO: 1 as a reverse primer. The length of the forward primer dose not have to be particularly limited and however, is typically 14 to 60 bases.  
30 Similarly, although the length of the reverse primer dose not have to be particularly limited, it is usually preferred that the length should be 14 to 60 bases. Examples of the forward and reverse primers used in the diagnostic method for cancer of the present invention include the followings:

the forward primer of 5'- GGAGCAGGAATTGGGGTCAC -3' (SEQ ID NO: 5);  
and

the reverse primer of 5'- TTGCTGTCCCATCCGGTGAG -3' (SEQ ID NO: 6).

When the detection is performed by the real-time quantitative polymerase chain  
5 reaction, examples of forward and reverse primers and a TaqMan probe used therein  
include the followings:

the forward primer of 5'- CCACTGTAGGCGCCCTAAGTT -3' (SEQ ID NO: 7);

the reverse primer of 5'- AAGAATGACCGGTGCAAGGA -3' (SEQ ID NO: 8);

and

10 the TaqMan probe of 5'- AAGGGCATCCCCCTGAGTCTTGGA -3' (SEQ ID  
NO: 9).

In the present invention, siRNA or siRNA corresponding to a polynucleotide  
comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof suppresses  
Nox1 gene expression and reduces Nox1 polypeptide production. Thus, the use of the  
15 siRNA or oligonucleotide will allow the inhibition of cell carcinogenesis or cancer  
progression induced by a mutant Ras gene.

The siRNA or oligonucleotide of the present invention refers to a nucleotide or an  
oligonucleotide complementary to a polynucleotide comprising a consecutive nucleotide  
sequence of SEQ ID NO: 1 or a fragment thereof.

20 The siRNA of the present invention may correspond to a polynucleotide comprising  
a nucleotide sequence at positions from 71 to 1615 of SEQ ID NO: 1 or a fragment thereof.  
The length of the siRNA is less than 100 bp and is preferably 8 to 99 bp, particularly  
preferably 10 to 30 bp. Moreover, preferable examples of the ssi include the followings:

siRNAs for human Nox1 of

25 5'-GCGTGGCTTCAGCATGGAATTCAAGAGATTCCATGCTGAAGCCACGCTTTTT  
TGGAAA-3' (SEQ ID NO: 10); and

5'-GGGCTTTCGAACAACAATATTCAAGAGATATTGTTGTTCGAAAGCCCTTTTT  
TGGAAA-3' (SEQ ID NO: 11); and

siRNAs for rat Nox1 of

30 5'-GTTATGAGAAGTCTGACAAGTTCAAGAGACTTGTCAGACTTCTCATAATTTT  
TTGGAAA-3' (SEQ ID NO: 12);

5'-GATTCTTGGCTAAATCCCATTCAAGAGATGGGATTAGCCAAGAATCTTTTT  
TGGAAA-3' (SEQ ID NO: 13); and

5'-GGACATTTGAACAACAGCATTCAAGAGATGCTGTTGTTCAAATGTCCTTTTT  
TGAAAA-3' (SEQ ID NO: 14).

The siRNA of the present invention can be used by directly administering the  
siRNA to a patient to be treated; by infecting the siRNA into a cell taken out of a patient to  
5 be treated and then returning the cell to the patient; or by administering an expression  
vector incorporating the siRNA therein to a patient to be treated and expressing the siRNA.

Thus, the present invention provides a pharmaceutical composition for cancer  
therapy, comprising: siRNA corresponding to a polynucleotide comprising the nucleotide  
sequence of SEQ ID NO: 1 or a fragment thereof; and siRNA corresponding to a  
10 polynucleotide comprising a nucleotide sequence at positions from 71 to 1615 of SEQ ID  
NO: 1 or a fragment thereof.

The pharmaceutical composition can be manufactured as the foregoing  
pharmaceutical composition comprising the antibody, by a routine method utilizing a  
pharmaceutical material conventionally used. The pharmaceutical composition  
15 comprising the siRNA of the present invention can be administered to, for example,  
humans or other mammals (e.g., rats, mice, guinea pigs, rabbits, sheep, pigs, cattle, horses,  
cats, dogs, and monkeys). The amount of the pharmaceutical composition administered  
appropriately differs depending on the conditions of objects to be administered,  
administration routes, and so on. For example, when orally administered, the  
20 pharmaceutical composition is generally administered at approximately 10 to 4000 mg,  
preferably approximately 20 to 2000 mg, more preferably approximately 50 to 500 mg, per  
day for an adult patient weighing 60 kg. When parenterally administered, it is preferred  
that the siRNA, for example in the form of injection, should be administered through the  
vein at a dose of approximately 10 to 2000 mg, preferably approximately 20 to 1000 mg,  
25 more preferably approximately 50 to 500 mg, per day for an adult patient weighing 60 kg  
although the dose differs depending on objects to be administered, the condition of cancer  
to be treated, and so on.

Moreover, the present invention provides a pharmaceutical composition for cancer  
therapy, comprising a cell transformed with siRNA corresponding to a polynucleotide  
30 comprising the nucleotide sequence of SEQ ID NO: 1 or a fragment thereof; and siRNA  
corresponding to a polynucleotide comprising a nucleotide sequence at positions from 71  
to 1615 of SEQ ID NO: 1 or a fragment thereof.

In this context, the cell to be transformed may be a cell donated from a third party or a cell taken out of a patient to be treated. Alternatively, the transformed cell can be used as a cell for cancer therapy.

Moreover, the present invention provides an RNA molecule consisting of a partial  
5 sequence of the nucleotide sequence of SEQ ID NO: 1, or an RNA molecule consisting of  
a mutant nucleotide sequence of the partial sequence with the addition, deletion, or  
substitution of at least one base, and suppressing Nox1 polypeptide expression in a human  
cell. The RNA molecule (siRNA) can be designed on the basis of the sequence of the  
polynucleotide of the present invention according to a method known in the art (e.g.,  
10 Nature, Vol. 411, pp. 494, 2001).

When the RNA molecule of the present invention is used in vivo or in vitro, it is  
used in the form of a double-stranded RNA molecule that consists of the RNA molecule  
and its complementary RNA. In this case, it is preferred that they should be treated so  
that the double-stranded RNA molecule is not degraded in a cell or is not dissociated into  
15 single strands. The treatment is performed by, for example, a method that adds a  
hydroxyl group to the 3' end of the RNA molecule, forms chemical bonds at both ends of  
the double strands through thiophosphoryl groups, or induces a chemical bond between  
both strands by ultraviolet radiation or through a bifunctional group such as  
bis(2-chloromethyl)amine, 4-thiouracil, or psoralen. The RNA molecule of the present  
20 invention suppresses Nox1 gene expression accompanying transformation with a mutant  
Ras gene and as such, can be used as a pharmaceutical composition for the prevention of  
cell carcinogenesis, the inhibition of cancer progression, and cancer therapy.

Moreover, the present invention provides a screening method for a cancer cell  
growth inhibitor targeted for a Nox1 gene. The screening method comprises bringing a  
25 cell having a mutant Ras gene or a normal cell transfected with a Nox1 gene into contact  
with a substance to be screened and then detecting the expression of the Nox1 gene and the  
inactivation of its enzyme activity. Subsequently, the transformed cell is cultured  
together with the substance to be screened to examine its ability to inhibit growth.

The influence of a compound to be screened on Nox1 gene expression can be  
30 examined by detecting an increase or decrease in mRNA expression by real-time  
quantitative polymerase chain reaction; by detecting a polypeptide or a peptide fragment  
thereof coded for the Nox1 gene with an antibody; or by observing morphological changes  
in the transformed cell.



The cell having a mutant Ras gene that can be used in the screening method, is not particularly limited, and examples thereof include H-Ras-NIH3T3 cells, K-Ras-NRK cells, and pancreatic cancer cells. For example, pEGFP-C1 (control) and pEGFP-C1-Nox1 can be used as a vector used in the introduction of the Nox1 gene, while for example, pSilencer  
5 can be used as an siRNA-expressing vector.

A cell line highly expressing Nox1 can be established by stably transfecting the pEGF-C1-Nox1 vector into an NIH3T3 or NRK cell by use of a Lipofectamine method. Alternatively, a pancreatic cancer cell having a mutant Ras gene can be utilized. It is preferred to use any of those separated and established from a human pancreatic cancer  
10 patient as the pancreatic cancer cell. These cells are cultured in a standard culture solution (Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS)) in the presence of 5% CO<sub>2</sub>. High-throughput screening can be conducted by culturing the cells in a multi-plate (96 or 48 wells), to which the substance to be screened is then added.

15 (Example 1)

Confirmation of Nox1 gene expression elevated by mutant Ras gene

Because Nox1 gene overexpression in NIH3T3 cells elevates superoxide generation and cell growth (1 and 2), the present inventors assumed that Nox1 is associated with the phenotypic expression of particular oncogenes. Therefore, cells transformed with K-Ras  
20 Val12 oncogene were used to confirm Nox1 gene expression elevated by Ras oncogene.

At first, rat kidney (NRK) cells and K-Ras-NRK cells (Kirstein murine sarcoma virus-transformed NRK cells) were prepared. The K-Ras-NRK cells were purchased from ATCC.

The presence or absence of Nox1 expression in the rat kidney (NRK) cells and the  
25 K-Ras-NRK cells was initially detected by real-time quantitative polymerase chain reaction. The real-time quantitative polymerase chain reaction used a forward primer of SEQ ID NO: 15, a reverse primer of SEQ ID NO: 16, and a TaqMan MGB probe of SEQ ID NO: 17.

Forward Primer:

30 5'- GGTCACCTCCCTTTGCTTCCA -3' (SEQ ID NO: 15)

Reverse Primer:

5'- GGCAAAGGCACCTGTCTCTCT -3' (SEQ ID NO: 16)

TaqMan MGB Probe:

5'- TCCAGTAGAAATAGATCTTT -3' (SEQ ID NO: 17)

The real-time quantitative polymerase chain reaction was performed using ABI Prism 7700 (Applied Biosystems) under reaction conditions set to defaults. Analytical values were standardized using rRNA 18S.

5 As a result of gene expression analysis by the real-time quantitative polymerase chain reaction, Nox1 gene expression was elevated in the K-Ras-NRK cells transformed with the K-Ras oncogene, as shown in the graph of Fig. 1(a).

(Example 2)

Confirmation of Nox1 gene expression elevated by transient transfection with mutant Ras  
10 gene

For analyzing the influence of transient transfection with mutant Ras gene, pcDNA3.1 vectors (blank vectors) and pcDNA3.1 carrying Ras Val12 vectors were used and transiently transfected into NRK cells. Forty-eight hours after the transfection, real-time quantitative polymerase chain reaction was conducted in the same way as in  
15 Example 1 in order to detect Nox1 expression. As a result, Nox1 gene expression was also elevated in the NRK cells transiently transfected with the H-Ras Val12, as compared with Nox1 gene expression in the control vector used, as shown in Fig. 1(b). The expression of the transfected Ras V-12 was confirmed by immunoblotting (IB) using a rabbit anti-Ras antibody.

20 For performing the immunoblotting,  $1 \times 10^5$  sample cells were solubilized with 2x sample buffers (0.1 M Tris-Cl (pH 6.8), 20% glycerol, 4% SDS, 3.1% DTT, and 0.001% BPB), and the polypeptides were separated by SDS gel electrophoresis and subsequently analyzed by the immunoblotting.

The proteins were electrically transferred to a nitrocellulose membrane in a transfer  
25 buffer (25 mM Tris-Cl (pH 8.3), 92 mM glycine, and 20% methanol) and reacted with an anti-Ras antibody (primary antibody) and an HRP-conjugated anti-rabbit-IgG antibody, followed by detection by a chemiluminescence (ECL) method.

As described above, the results of Examples 1 and 2 indicated that Nox1 gene expression is elevated by the action of mutant Ras oncogene.

30 NIH3T3 cells cultured overnight under serum-free conditions were treated mitogenically with serum (30%) or 50 ng/ml epidermal growth factor (EGF) and then analyzed by real-time quantitative polymerase chain reaction. As a result, Nox1 gene expression was increased to 6 to 20 times within 12 hours from the initiation of the culture,

as shown in Figs. 1(c) and (d). That is, Nox1 gene expression is associated with factors that promote cell growth. This is consistent with the reports saying that both platelet-derived growth factor (PDGF) and angiotensin II induce superoxide formation and Nox1 gene expression in vascular smooth muscle cells (1 and 4).

5 (Example 3)

Analysis of signaling pathway of Nox1 gene expression caused by mutant Ras gene

A series of experiments were performed for analyzing the downstream region of a Ras signaling pathway of Nox1 gene expression elevated by mutant Ras gene. In Examples 1 and 2, the cells were treated for 12 hours with PD98059 (PD: 20  $\mu$ M, 100  $\mu$ M),  
10 an MAPKK inhibitor, to analyze Nox1 expression in the K-Ras-NRK cells and serum- and EGF-induced Nox1 expression by real-time quantitative polymerase chain reaction.

As a result, Nox1 expression in the K-Ras-NRK cells was inhibited by the PD98059 concentration-dependently (Fig. 1(a)). Moreover, serum- and EGF-induced Nox1 expression was respectively inhibited by 20  $\mu$ M PD98059 (Figs. 1(c) and (d)).

15 On the other hand, wortmannin (100 nM), a PI3 kinase inhibitor, did not inhibit an increase in Nox1 expression in the H-Ras-NIH3T3 cells in a control experiment (data not shown). These results indicated that the signals of mutant Ras gene and growth factors thereof induce Nox1 expression, by pathways not via PI3K but via Ras-MAPKK-MAPK. (Example 4)

20 Involvement of Nox1 gene in transformation with mutant Ras gene

Oligonucleotides 1-S and 1-AS were annealed and subcloned into a pSilencer hygromycin vector with an H1-promoter (Ambion) to yield RNAi(1). Likewise, oligonucleotides 3-S and 3-AS were annealed and subcloned into a pSilencer hygromycin vector with an H1-promoter (Ambion) to yield RNAi(3). In addition, oligonucleotides 5-S and 5-AS were annealed  
25 and subcloned into a pSilencer hygromycin vector with an H1-promoter (Ambion) to yield RNAi(5). A pSilencer hygromycin Negative Control plasmid (Ambion) was used as a control vector.

The RNAi(1) is an siRNA construction targeted for positions from 223 to 241 of SEQ ID NO: 3. The RNAi(3) is an siRNA construction targeted for positions from 578 to  
30 596 of SEQ ID NO: 3. The RNAi(5) is an siRNA construction targeted for positions from 1224 to 1242 of SEQ ID NO: 3.

The sequence of each oligonucleotide is as follows:

1-S:

5'-GATCCCGTTATGAGAAGTCTGACAAGTTCAAGAGACTTGTCTCAGACTTCTCAT  
AATTTTTTGGAAA-3' (SEQ ID NO: 18)

1-AS:

5'-AGCTTTTCCAAAAAATTATGAGAAGTCTGACAAGTCTCTTGAAGTTGTCAGA  
5 CTTCTCATAACGG-3' (SEQ ID NO: 19)

3-S:

5'-GATCCCGATTCTTGGCTAAATCCCATTCAGAGATGGGATTTAGCCAAGAAT  
CTTTTTTGGAAA-3' (SEQ ID NO: 20)

3-AS:

10 5'-AGCTTTTCCAAAAAAGATTCTTGGCTAAATCCCATCTCTTGAATGGGATTTA  
GCCAAGAATCGG-3' (SEQ ID NO: 21)

5-S:

5'-GATCCCGGACATTTGAACAACAGCATTCAAGAGATGCTGTTGTTCAAATGTC  
CTTTTTTGGAAA-3' (SEQ ID NO: 22)

15 5-AS:

5'-AGCTTTTCCAAAAAAGGACATTTGAACAACAGCATCTCTTGAATGCTGTTGT  
TCAAATGTCCGG-3' (SEQ ID NO: 23)

4 µg each of the RNAi(1), RNAi(3), RNAi(5), and pSilencer hygro Negative  
Control plasmid was transfected respectively into  $1 \times 10^6$  K-Ras-NRK cells using  
20 Lipofectamine (Gibco-BRL). The transfected cells were cultured for selection at 37°C  
for 2 to 3 weeks in a DMEM medium supplemented with 10% fetal bovine serum (FBS)  
and 400 µg/ml hygromycin B under a 5% CO<sub>2</sub> moisture environment. After the culture,  
surviving colonies were isolated.

Three clones of the cell lines stably transfected with each of the RNAi(1), RNAi(3),  
25 and RNAi(5) (K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(1)-12, and  
K-Ras-NRK/RNAi(1)-15; K-Ras-NRK/RNAi(3)-17, K-Ras-NRK/RNAi(3)-19, and  
K-Ras-NRK/RNAi(3)-96; and K-Ras-NRK/RNAi(5)-2, K-Ras-NRK/RNAi(5)-3, and  
K-Ras-NRK/RNAi(5)-7) as well as two clones of the cells stably transfected with the  
pSilencer hygro Negative Control plasmid (K-Ras-NRK/neg-1 and K-Ras-NRK/neg-2)  
30 were selected. The transfection of these constructions was confirmed by PCR using  
M13F and 3.0Rev as primers (Fig. 2). PCR temperature conditions were set to 94°C for 2  
minutes, 30 cycles of (94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute),

followed by 72°C for 10 minutes. A thermal cycler used was Takara Thermal Cycler SP (Takara Shuzo Company Limited).

(Primer Sequence)

M13F: 5'- GTTTCCCAGTCACGAC-3' (SEQ ID NO:24)

5 3.0Rev: 5'-GAGTTAGCTCACTCATTAGGC-3' (SEQ ID NO: 25)

Next, cellular morphological changes in which Nox1 gene was involved were compared among the cells. As a result, as shown in Fig. 3, the K-Ras-NRK cells transfected with the RNAi(1) to RNAi(3) were extended, whereas the K-Ras-NRK cells transfected with the pSilencer hygro Negative Control plasmid morphologically remained  
10 round as the cells observed before introduction.

Subsequently, soft agar culture assay was performed in order to examine the transformation of the cells by observing anchorage-independent growth capacity. An agarose layer containing 0.53% agar and nutrients necessary for cell growth was placed and solidified in a culture dish of 6 cm in diameter, on which the cells suspended in a  
15 DMEM containing 0.3% agar and 10% FBS were then piled up at a final concentration of  $1.5 \times 10^4$  cells/culture dish. Subsequently, the cells were cultured at 37°C under a 5% CO<sub>2</sub> moisture environment to observe the appearance of cell colonies over 3 weeks. The adhesion-dependent cell growth of the cell lines exhibited by the soft agar culture was measured, and +/- average standard deviation (s.e.m.) from three measurements was shown  
20 in Fig. 4. As a result, the formation of many colonies was observed in the K-Ras-NRK cells and the transfected cells with the pSilencer hygro Negative Control plasmid (neg-1 and neg-2), whereas colony formation was remarkably inhibited in the transfection with the RNAi(1) to RNAi(5).

In addition,  $10^4$  cells each of the K-Ras-NRK/neg-1 (neg-1),  
25 K-Ras-NRK/RNAi(1)-7 (i(1)-7), K-Ras-NRK/RNAi(3)-19 (i(3)-19), and K-Ras-NRK/i(5)-7 (i(5)-7) cells were transferred from the culture dish to a culture solution and then liquid-cultured for 6 days in a standard culture solution (DMEM containing 10% FBS) in the presence of 5% CO<sub>2</sub>. Subsequently, the number of the cells in the liquid medium was measured, and its growth curve was shown in Fig. 5. As a result, each  
30 growth rate of the K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(3)-19, and K-Ras-NRK/RNAi(5)-7 cells was also decreased in the liquid culture, and by contrast, inhibitory effect on growth was not observed in the K-Ras-NRK/neg-1.

(Example 5)

Confirmation of superoxide production by NADPH oxidase coded for Nox1 gene

The present inventors measured superoxide production by NBT reduction analysis using the transformed cells of Example 4 in order to evaluate the role of Nox1 in superoxide production induced by mutant Ras. In the present example, the method of Suh et al. was used in the NBT (nitroblue tetrazolium) analysis (1). Namely, 0.2 ml of a Hanks solution containing 0.25% NBT was prepared with or without 40 units of superoxide dismutase (SOD), a reactive oxygen-digesting enzyme, in which  $2 \times 10^5$  cells were in turn suspended and treated at 37°C for 8 minutes. The treated cells were separated by low-speed centrifugation and solubilized by the addition of 0.5 ml of pyridine. Absorbance at 510 nm was measured to quantify a decrease in NBT (extinction coefficient of 11,000/M/cm). The obtained data was shown in Fig. 6 with +/-s.e.m. from 3 measurements.

As a result of the analysis, the K-Ras-NRK and K-Ras-NRK/neg-1 increased NBT reduction, which was inhibited by the superoxide dismutase treatment, as compared with the NRK. By contrast, the K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(3)-19, and K-Ras-NRK/RNAi(5)-7 decreased the ROS production-stimulating effect of K-Ras oncogene to the level of the NRK cells (Fig. 6), thereby indicating that Nox1 is involved in an increase in superoxide production in mutant Ras-transformed cells. (Example 6)

Confirmation of Nox1 expression decreased by RNAi(1), RNAi(3), and RNAi(5)

The present inventors conducted analysis in the following procedures in order to confirm Nox1 expression decreased by the RNAi(1), RNAi(3), and RNAi(5): GFP-rat Nox1 was subjected to cotransfection with each of the RNAi(1) to (5) and subsequently immunoblotting analysis was conducted in the same way as in Example 2 in order to evaluate the inhibitory effect of the RNAi(1), RNAi(3), and RNAi(5) on GFP-rat Nox1 expression. As shown in the left panel of Fig. 7, it was revealed that an expected GFP-rat Nox1 fusion polypeptide is produced, and that the coexpression of the RNAi(1), RNAi (3), and RNAi(5) suppresses the production of a GFP-rat Nox1 fusion protein dependently on a dose of these vectors. GFP-Nox1 expression shown in the left panel of Fig. 7 was quantitatively determined by immunoblotting using an anti-GFP antibody. Numerals in the left panel of Fig. 7 denote the amount ( $\mu$ g) of DNA transfected. Both RNAi(1) and RNAi(5) are targeted for rat Nox1. The same approach as in the left panel of Fig. 7 was

used to show in the right panel of Fig. 7 that the RNAi(1) and RNAi(5) do not inhibit human Nox1, that is, the RNAi constructions have high specificity to their target gene.

Moreover, RT-PCR demonstrated that the mRNA expression of endogenous Nox1 is certainly inhibited in each of the K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(1)-12,

5 K-Ras-NRK/RNAi(3)-19, K-Ras-NRK/RNAi(3)-96, K-Ras-NRK/RNAi(5)-2, K-Ras-NRK/RNAi(5)-7 cells, as compared to the K-Ras-NRK and K-Ras-NRK/neg-1 cells. The result is shown in Fig. 8.

Sequences of PCR Primers used in Example 6

Forward Primer: 5'-ATGGGAACTGGCTGGTTA-3' (SEQ ID NO: 26)

10 Reverse Primer: 5'-TCAGAACGTTTCTTTGTTGAA-3' (SEQ ID NO: 27)

The results of the present Example indicate that an increase in the expression rate of Nox1 gene and transformation with mutant Ras are associated with each other, as well as morphological changes caused by the constitutional changes of cytoskeletons and adhesive proteins.

15 (Example 7)

Influence of reactivation of Nox1 once inhibited by siRNA on cell

pEGFP-C1 (GFP) and pEGFP-human Nox1 (GFP-Nox1) were separately transfected into the K-Ras-NRK/RNAi(1)-7 and K-Ras-NRK/RNAi(5)-7 by the same approach as in Example 4, then immunoblotting was conducted by the same approach as in

20 Example 6 (Fig. 9). As shown in Example 6 and the right panel of Fig. 7, neither RNAi(1) nor RNAi(5) inhibits human Nox1 expression. Therefore, even if the pEGFP-human Nox1 is transfected into the K-Ras-NRK/RNAi(1)-7 and K-Ras-NRK/RNAi(5)-7 cells, this human Nox1 is not inhibited by siRNA.

As shown in Fig. 10, the K-Ras-NRK/RNAi(1)-7 cells could not derepress siRNA and were oblate only by introducing the control vector therein (i(1)-7+GFP), whereas they morphologically returned to the spherical shape close to the original K-Ras-NRK cells by transfecting the pEGFP-human Nox1 therein (i(1)-7+GFP-Nox1-3) (Fig. 10).

Growth curves obtained by the same approach as in Example 4 also exhibited accelerated growth in the pEGFP-human Nox1-incorporated K-Ras-NRK/RNAi(1)-7

30 (GFP-Nox1-3) and pEGFP-human Nox1-incorporated K-Ras-NRK/RNAi(5)-7 (GFP-Nox1-11), as compared with the control vector-incorporated K-Ras-NRK/RNAi(1)-7 (GFP-59) and the control vector-incorporated K-Ras-NRK/RNAi(5)-7 (GFP-78) (Fig. 11). Moreover, the soft agar culture assay in the same way as in Example 4 also demonstrated

that colony formation in the pEGFP-human Nox1-incorporated K-Ras-NRK/RNAi(1)-7 (GFP-Nox1-2 and GFP-Nox1-3) recovered to the same level as that in the original K-Ras-NRK cells, as compared with the control vector-incorporated K-Ras-NRK/RNAi(1)-7 (i(1)-7+GFP-59 and i(1)-7+GFP-60) (Fig. 12).

5 (Example 8)

Influence of Nox1 gene expression on transformation with mutant Ras

Diphenylene iodonium (DPI), a Nox1 inhibitor, was used to examine the influence of Nox1 gene expression on the morphologies of the cells transformed with mutant Ras. Namely, K-Ras-NRK cells and NRK cells were cultured overnight at 37°C in a medium  
10 containing 20  $\mu$ M DPI or 30  $\mu$ M PD98059 under a 5% CO<sub>2</sub>-containing moisture environment to observe morphological changes. Photographs documenting the cell morphologies are shown in Fig. 13.

As shown in Fig. 13, the K-Ras-NRK cells transiently assumed a flat shape that was close to the shape of the NRK cells, when the K-Ras-NRK cells were treated overnight  
15 with flavoprotein inhibitor DPI or 10 mM antioxidant n-acetyl cysteine (data not shown). As further shown in Fig. 13, such morphological changes were also observed in the K-Ras-NRK cells treated with the PD98059. Thus, these results indicated that an increase in Nox1 gene expression is essential to Ras-MAPKK-MAPK pathway-promoted transformation with mutant Ras.

20 (Example 9)

Influence of siRNA on tumor formation caused by mutant Ras

The K-Ras-NRK/neg-1, K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(1)-12, K-Ras-NRK/RNAi(3)-19, K-Ras-NRK/RNAi(3)-96, and K-Ras-NRK/RNAi(5)-2 cells were separately transplanted into athymic mice to observe tumor formation. Namely, 10<sup>6</sup>  
25 cells each of the cells were suspended in 0.2 ml PBS and then subcutaneously transplanted into the nude mice (athymic: Nu/Nu). Then, tumor formation in all of the nude mice was observed over 1 month, while the volume of the formed tumor was measured. The result was shown in Fig. 14. In Fig. 14, the solidly shaded bars denote the volume of tumor; the error bars denote s.e.m.; and the fractions denote the proportion  
30 of tumor-bearing mice to all the mice used (tumor/total).

As shown in Fig. 14, the K-Ras-NRK/neg-1 formed active tumor within 2 weeks. By contrast, the K-Ras-NRK/RNAi(1)-7, K-Ras-NRK/RNAi(1)-12, K-Ras-NRK/RNAi(3)-19, and K-Ras-NRK/RNAi(3)-96 remarkably suppressed tumor



growth. When histologically observed by anatomy, the tumor formed in the K-Ras-NRK/neg-1 resulted in increased blood vessels. This is consistent with the possibility that Nox1 gene induces angiogenesis caused by increase in vascular endothelial growth factor production (1). The small tumor formed in the K-Ras-NRK/RNAi(1)-7,  
5 K-Ras-NRK/RNAi(1)-12, K-Ras-NRK/RNAi(3)-19, and K-Ras-NRK/RNAi(3)-96 exhibited signs of necrosis in most of the cells (data not shown).

As seen from these experimental results, mutant Ras (oncogene) elevates mitogenic oxidase Nox1 via MAPKK-MAPK pathways, whereas Nox1 gene expression is essential to transformation of cells, cancer formation, and its progression caused by the Ras.

10 Namely, the studies conducted by the present inventors revealed a molecular mechanism in which a Nox1 polypeptide plays an essential role as a redox signal molecule in transformation with mutant Ras.

Nox family proteins Nox1 to 5, Gp91phox homologues, were identified from non-phagocytic cells and reported to respectively have specific functions in various cell  
15 processes (1 and 5 to 9). Among the Nox family, the Nox1 gene is unique in that it codes for transmembrane oxidase that mediates the mitogenic signals from growth factors (1 and 4) and oncogenes.

Considering the involvement of ROS in the promotion of tumor growth and the possibility of ROS production for inducing ROS-producing enzymes in malignant cancer  
20 cells (7), Nox1 gene and its polypeptides are probably capable of suppressing cancer progression and serving as target molecules that allow the cancer therapy.

The SEQ ID NOs in the sequence listing of the present specification represent the following sequences:

SEQ ID NO: 1 represents the nucleotide sequence of mRNA transcribed from a  
25 human Nox1 gene or cDNA;

SEQ ID NO: 2 represents the amino acid sequence of a polypeptide coded by the human Nox1 gene;

SEQ ID NO: 3 represents the nucleotide sequence of mRNA transcribed from a rat Nox1 gene or cDNA;

30 SEQ ID NO: 4 represents the amino acid sequence of a polypeptide coded by the rat Nox1 gene;

SEQ ID NO: 5 represents the nucleotide sequence of a forward primer corresponding to a Nox1 gene used in a diagnostic method for human cancer;

SEQ ID NO: 6 represents the nucleotide sequence of a reverse primer corresponding to a Nox1 gene used in a diagnostic method for human cancer;

SEQ ID NO: 7 represents the sequence of a forward primer used in the detection of a human Nox1 gene by real-time quantitative polymerase chain reaction;

5 SEQ ID NO: 8 represents the sequence of a reverse primer used in the detection of a human Nox1 gene by real-time quantitative polymerase chain reaction;

SEQ ID NO: 9 represents the sequence of a TaqMan probe used in the detection of a human Nox1 gene by real-time quantitative polymerase chain reaction;

10 SEQ ID NO: 10 represents the nucleotide sequence of siRNA for a human Nox1 gene of the present invention;

SEQ ID NO: 11 represents the nucleotide sequence of siRNA for a human Nox1 gene of the present invention;

SEQ ID NO: 12 represents the nucleotide sequence of siRNA for a rat Nox1 gene of the present invention;

15 SEQ ID NO: 13 represents the nucleotide sequence of siRNA for a rat Nox1 gene of the present invention;

SEQ ID NO: 14 represents the nucleotide sequence of siRNA for a rat Nox1 gene of the present invention;

20 SEQ ID NO: 15 represents the nucleotide sequence of a forward primer used in Example 1 for detecting the presence or absence of Nox1 expression by real-time quantitative polymerase chain reaction;

SEQ ID NO: 16 represents the nucleotide sequence of a reverse primer used in Example 1 for detecting the presence or absence of Nox1 expression by real-time quantitative polymerase chain reaction;

25 SEQ ID NO: 17 represents the nucleotide sequence of a TaqMan MGB probe used in Example 1 for detecting the presence or absence of Nox1 expression by real-time quantitative polymerase chain reaction;

30 SEQ ID NO: 18 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

SEQ ID NO: 19 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

SEQ ID NO: 20 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

5 SEQ ID NO: 21 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

SEQ ID NO: 22 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

10 SEQ ID NO: 23 represents the nucleotide sequence of an oligonucleotide that constitutes an siRNA construction used in Example 4 for examining the involvement of a Nox1 gene in transformation with a mutant Ras gene;

SEQ ID NO: 24 represents the nucleotide sequence of an M13 primer used in Example 4 for confirming the transfection of the siRNA constructions for a Nox1 gene;

15 SEQ ID NO: 25 represents the nucleotide sequence of a 3.0 Rev primer used in Example 4 for confirming the transfection of the siRNA constructions for a Nox1 gene;

SEQ ID NO: 26 represents the nucleotide sequence of a forward primer used in Example 6 for confirming a decrease in Nox1 expression; and

20 SEQ ID NO: 27 represents the nucleotide sequence of a reverse primer used in Example 6 for confirming a decrease in Nox1 expression.

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